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Elevated body weight or body mass index and weight gain have been proposed to play a role in the development of postmenopausal breast cancer. How body weight plays a role in breast cancer etiology is unknown. Animal models are frequently used to study human diseases. Although few in number published studies have reported increased body eight associated with higher incidences of either chemically-induced or spontaneous mammary tumors. Additional publications have implicated weight gain in the development of chemically-induced mammary cancer. In the present studies, we propose that the role of body weight and weight gain in the development of breast cancer can be addressed in a physiologically relevant animal model, *i.e.*, transgenic mice. "Hybrid" obese transgenic mice are being produced by mating strains of genetically obese mice in which the molecular defect has been identified, *i.e.*, Lep^{ob} and $Lepr^{db}$, with a transgenic mouse line overexpressing TGF-α. This oncogene produces mammary tumors in mice and has been implicated in the etiology of human breast cancer. These hybrid mice are being used to systematically evaluate the role of body weight and weight gain in the development of mammary tumors in genetically obese, dietary obese, and lean mice.

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Margot P Cleary 9/28/98

PI - Signature Date

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INTRODUCTION:

Breast cancer is the most frequently diagnosed cancer in women (1). One risk factor that has been proposed to play a role in the development of postmenopausal breast cancer is increased body weight and/or body mass index (BMI) (weight/height^2). Several reviews of the literature have shown that both case-control and prospective studies support this conclusion (2,3). A recent study of Italian women concluded that overweight accounted for 10.2% of postmenopausal breast cancer cases (4). Other studies have implicated weight gain as a risk factor for breast cancer development (5-8). In spite of these reports, roles for body weight and weight gain in breast cancer development have not been supported by all studies. This may be partially explained by technical factors. For example, conflicting results from human studies might be due to inaccurate recalled body weight. Also, whether weights from before or after menopause are used appears to make a difference in the conclusions drawn (9). In addition to these problems, there are other confounding factors such as the influences of ethnic and social backgrounds, and the potential for interactions with other risk factors.

Animal models are frequently used to study human diseases. Currently, the number of published studies investigating the role of obesity and mammary tumorigenesis in rodents is limited (10-17). Strikingly, however, in all these studies, obese rodents have been found to have a higher incidence of either chemically-induced or spontaneous mammary tumors. Two additional publications using weight-cycled rats further implicate weight gain as an important factor in the development of chemically-induced mammary tumors (18,19). Clearly, the role of these factors as potential risk factors in the etiology of human breast cancer is an important issue to resolve.

We have proposed that the role of body weight, BMI and weight gain in the development of breast cancer can be addressed systematically in a physiologically relevant animal model, *i.e.*, transgenic mice. Our hypothesis is that weight gain and the accompanying metabolic changes create a milieu conducive to enhanced development of oncogene-induced mammary tumors. We are developing a molecularly well-defined animal model to test this hypothesis. "Hybrid" obese-transgenic mice are being produced by mating strains of genetically obese mice in which the molecular defect has been identified, *i.e.*, *Lep*^{ob} or *Lepr*^{db}, with a transgenic mouse line overexpressing TGF-α. This proto-oncogene has been implicated in the etiology of human breast cancer (20,21), and its presence in mice has been demonstrated to result in mammary tumors (22). These hybrid mice are being used to systematically evaluate the role of body weight and weight gain in the development of mammary tumors in genetically obese, dietary obese, and lean mice.

BODY:

Our goals for year one of this project as outlined in the Statement of Work (see Appendix A) were to 1) set up genotyping assays for TGF-α and *ob*, 2) create the hybrid TGF-α-*ob* mice, and 3) enter experimental mice into the incidence study. All of these goals have been met.

Experimental Methods:

Genotyping Assays.

a) DNA extraction. The first step for any genotyping assay is the preparation of the DNA samples. We obtain small samples from the tails of mice shortly after weaning. DNA is isolated from tail samples using PBND and Proteinase K at 55°C. Samples are extracted with phenol:isoamylalcohol and then washed with alcohol. Samples are dried and dissolved in TE buffer. DNA concentration is determined spectrophotometrically, and samples aliquoted for PCR genotyping assays.

b) PCR-MMTV-TGF-α assay. Mice are identified by either the absence or presence of MMTV-TGF-α. Single stranded PCR amplification protocol is used to amplify a portion of the TGF-α from genomic DNA using two sets of oligonucleotide primers designed by Jackson laboratory. Primer 199 (5'GATCTTTTCTATGGAATAAGGAATGGA) corresponds to a region of the MMTV vector just upstream of the TGF-α insert while the complimentary primers 200 (5'- CTAGGCCACAGAATTGAAAGATCT) and 043 (5'GTAGGTGGAATTCTAGCATCATCC) are specific for a mouse sequence outside of the inserted MMTV-TGF-α gene and are used as an internal control, with a predicted product size of 324bp. Thermal cycler parameters involve a modified step-down technique with a 30 sec denaturation at 94°C and a 1 min anneal at 3°C intervals starting at 67°C and finishing at 52°C, followed by a 2 min extension at 72°C. Three cycles at each step were performed with 20 cycles on the final step. The PCR product sizes were verified by agarose gel electrophoresis.

c) PCR- *ob/ob* assay. This protocol was established to distinguish both homozygous (Lep^{+}/Lep^{+}) and heterozygous (Lep^{+}/Lep^{ob}) lean mice, and homozygous (Lep^{ob}/Lep^{ob}) obese mice. In general, this assay is performed in a similar manner as that described above for TGF-α. However, a different set of primers and an additional restriction digest of the PCR product are used. Primer OB1 (5'TGTCCAAGATGGACCAGACTC) along with complimentary OB2 (5'ACTGGTCTGAGGCAGGAGCA) amplify a region of the *ob* gene with an expected product of 155bp. This product includes a DdeI restriction site only in samples containing the *ob* gene. Agarose gel electrophoresis following DdeI digestion distinguishes heterozygous lean mice from homozygous obese mice.

Procedures:

Mating strategies. Initially, we proposed to use both male and female mice that carried the transgene, TGF-α, and were heterozygous for *ob*, *i.e.*, Lep^{+}/Lep^{ob}, in the mating protocol. However, we discovered that the TGF-α female mice although fertile do not successfully lactate. We tried cross fostering the pups to nontransgenic lactating females, but pup mortality was very high. Therefore, we changed our strategy to use nontransgenic female mice for breeding and lactation. Male TGF-α heterozygous (Lep^{+}/Lep^{ob}) mice are bred with nonTGF-α-Lep^{+}/Lep^{ob} female mice to produce offspring with or without the transgene, and that are either homozygous lean, heterozygous lean, or homozygous obese. We have found that as expected this provides more heterozygous offspring than

either of the two homozygous genotypes. Thus, we are now including two additional breeding strategies to increase our production of homozygous lean and obese offspring. One is to breed both male and female mice that are homozygous lean. In addition, we are treating obese male mice with leptin to restore fertility. This approach was based on a previous study (23) where is was reported that treatment of adult (70 g) mice with 20 μg of leptin per g of body weight resulted in successful pregnancy in females mice housed with the treated male mice. We used a similar approach in younger mice but injected a much lower dose of leptin. In the first study two, 10-week-old male obese mice were treated with 5 μg of leptin per g of body weight for 16 days and then with 2.5 μg of leptin per g of body weight for 8 additional days. One experienced female mouse was placed with the each male mouse 3 days after initiating leptin treatment and an additional female mouse was added after 3 days. The female mice were removed after 6-7 days so that after the first 3 days with the initial female there were always two female mice in the cage. The last females added only had two-day exposure to the male mice before the study was terminated. In the second study, four, 8-10-week-old male mice were treated with 2.5 μg of leptin per g of body weight for 38-44 days. Pairs of females were provided to each of the four male mice for 7-day periods after the first week of treatment. Heterozygous lean females are used to result in an expected 50% obese offspring.

Mice. Small pieces of tails are removed from the mice at 4-6 weeks of age. After DNA extraction the samples are assayed to determine the animal's TGF and/or *ob* status. Dependent upon sex and genotype results mice are assigned to the breeding colony, the experimental study, or to be euthanized.

Results:

Leptin Treatment. Both male mice from Study 1 lost weight over the course of the experiment. One mouse went from 42.5 g to 29.6 g, and the second from 41.4 g to 29.0 g. Neither of the first females placed with the male mice became pregnant. One of the males impregnated the remaining 5 females he was housed with. The other male mouse fathered a litter with the 3rd, 5th, and 6th females. In the second experiment the mice also lost weight (37.9g→ 29.3 g). Three of the four mice treated with the 2.5 μg leptin dose impregnated females (4/10, 5/6, and 2/6 {# pregnancies/# female mice}). Vehicle-treated control mice were also placed with females, but as expected no pregnancies resulted.

TGF-α *ob* mice. As of August 31,1998 we have produced a total of 228 litters, and 1296 mice have been genotyped. 23 obese TGF-α-Lep^{ob}/Lep^{ob} mice (oldest mouse 33 wk), 41 lean heterozygous TGF-α Lep^{+}/Lep^{ob} mice (oldest 42 wk), and 27 lean homozygous TGF-α Lep^{+}/Lep^{+} mice (oldest 40 wk) have been assigned to the incidence study. To date no tumors have been detected. Although we initially planned to only enroll the two homozygous groups in the study, we have now included the heterozygous lean mice. This was done for two reasons. First, we had planned to use the heterozygous mice for mating but due to the lactation problem this is no longer feasible. Second, there was a recent report (24) that there is a heterozygous effect of this gene that affects body weight, body fat, and hormone levels. Interestingly, if one looks at body weight curves for our mice it appears that this effect is apparent (Figure 1). In order to obtain adequate blood

samples and not interfere with our ongoing study we have decided to use non-transgenic female mice for blood sampling. To date 10 obese, 7 heterozygous lean and 9 homozygous female mice have been saved for this purpose.

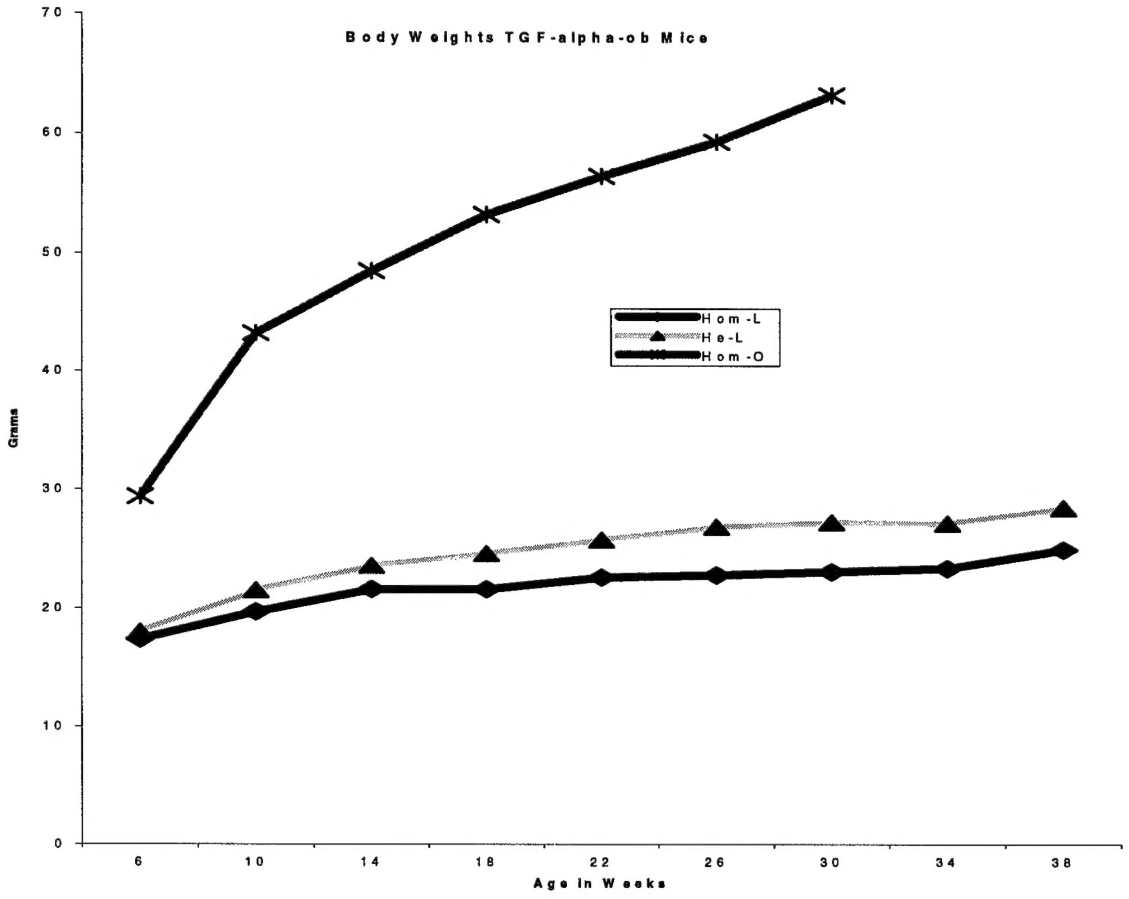

Figure 1. Body weight curves for TGF-α female mice.

Body weights of TGF-α female mice enrolled in the Incidence study. Hom-L = homozygous Lep^{+}/Lep^{+} mice, He-L = heterozygous Lep^{+}/Lep^{ob} mice, and Hom-O = homozygous Lep^{ob}/Lep^{ob} mice. Numbers per group range from 5-39 dependent upon age and group.

We will begin enrolling heterozygous lean mice in the weight-cycling study. The diets have been bought.

TGF-α *db* mice. A recent paper (25) indicated that the *db* mouse strain that we had planned to buy from Jackson Laboratory could potentially have growth problems related to the gene for misty. After having spoken with Dr. Gary Truett and evaluating the information on hand, we have decided to use $Lepr^{db}/Lepr^{db}$ without the misty gene that Dr. Truett and his coworkers have developed. These mice arrived several weeks ago and breeding of the *db* mice has been initiated. Dr. Truett also provided an assay to genotype the mice and this is being set up in the laboratory.

Discussion:

Statement of Work: With respect to our statement of work we are on schedule. We have almost finished recruitment into the Incidence study and will begin enrolling mice in the weight-cycling protocol.

Our findings with the leptin-treated male mice are of general interest and should lead to a publication as soon as we have completed study on several additional mice.

Due to the long-term nature of these experiments, we have limited data to present at this time.

CONCLUSIONS:

This research project is going along very well. Although we have had a few setbacks, we have redefined our strategy and dealt with them. This relates to the inability to use the transgenic female mice for breeding. This in turn has resulted in our having to breed twice as many mice as we had originally planned, and we also must genotype twice as many offspring. However, we have dealt with these problems and still have stayed on our time line.

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III.B.2.c. Statement of Work

Months 1-2. Order breeding animals, racks and initial supplies. Set up assays for trans-oncogene and *ob* determinations.

Months 3-5. Initiate first set of breedings and determine genotype of offspring.

Months 5-6. Initiate matings of double heterozygous mice. Enroll female homozygous lean (+/+) and obese (*ob/ob*) mice in breast cancer incidence study. Test for genotypes and take blood samples. Weigh experimental mice.

Months 6-12. Continue double heterozygous matings and start homozygous (**TT**) trans-oncogene matings. Continue to enroll female homozygous lean (+/+) and obese (*ob/ob*) mice from both mating groups in the incidence study. Test for genotypes and take blood samples. Weigh mice and examine for tumors weekly. Monitor tumor growth.

Months 13-18. Continue to monitor mice enrolled in the incidence study. Collect samples from ethanized mice. When 25 mice enrolled per lean and obese groups, inititate weight-cycling study.
Monitor those mice, take blood samples and prepare and feed special diets.

Months 15-18. Order *db* mice and inititate that breeding colony. Enroll *ob* mice in weight-cycling study and record weekly weights and food intakes and monitor tumor incidenc and growth in these mice. Prepare and feed special diets.

Months 18-21. Initiate matings of double heterozygous trans-oncogene *db* mice. Enroll female homozygous lean (+/+) and obese (*db/db*) mice in breast cancer incidence study. Test for genotypes and take blood samples. Weigh and monitor experimental mice and continue monitoring food intakes of weight-cycled mice. Continue diet preparations. Kill mice from the incidence study as they reach 16 months of age. Do body compositions on mice from incidence study. Enroll *ob* strain mice in diet-induced obesity study and monitor.

Montha 22-28. Continue heterozygous matings and start homozygous (**TT**) trans-oncogene matings of *db* mice colony. Continue to enroll *db* strain in incidence study and weigh mice, examine for tumors and monitor tumor growth of all experimenatal mice. Kill weight-cycled mice as they reach 16 months of age. Do cellularity and body compostion determinations. Kill mice from db incidence study and enroll additional animals in weight-cycling protocol.

Months 24-36. Record food intakes, body weight and monitor for tumors and tumor growth. Kill remaing weight-cycled mice and kill diet-induced obese *ob* mice as they reach 16 months of age. Perform cellularity measurements and body compostition analysis. Kill *db* mice from incidence study.